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Is there any still undisclosed biodiversity in *Ciauscolo* salami? A new glance into the microbiota of an artisan production as revealed by high-throughput sequencing

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Abstract

Ciauscolo is a fermented sausage with the Protected Geographical Indication (PGI) status. To disclose the microbial ecology of a model *Ciauscolo* salami manufacture during its natural fermentation, viable counting, amplicon-based sequencing and PCR real-time were applied. The volatilome during fermentation was also characterized. The results allowed previously undetected species to be discovered. The core microbiota was composed by *Lactobacillus algidus*, *Leuconostoc carnosum*, *Lactobacillus sakei*, *Debaryomyces hansenii*, *Glomus hyderabadensis*, *Tilletiopsis washingtonensis*, and *Kurtzmaniella zeylanoides*. *Salmonella* spp. and *Listeria monocytogenes* were absent in all the samples; moreover, multiplex real-time PCR revealed the absence of the target genes *bont/A*, *bont/B*, *bont/E*, *bont/F*, and *4gyrB (CP)*, encoding botulinic toxins. Volatilome, deeply depending on microbiological metabolism, was characterized by spices-derived components. Limonene, sabinene, α- and β-pinene, 3-carene, and α-thujene were the most represented monoterpene hydrocarbons, whereas β- and α-copaene were the most represented sesquiterpene hydrocarbons. Allyl methyl sulphide and diallyl disulphide were the major aliphatic sulphur compounds, together with diallyl sulphide and allyl methyl disulphide.

Keywords: *Lactobacillus algidus*, *Luconostoc carnosum*, *Kurtzmaniella zeylanoides*, volatilome, Illumina sequencing

1. Introduction

The art of meat preservation, by salting and drying, dates back to the ancient Egypt and was particularly widespread among the Greeks, the Romans, the Gauls, the Celts and the Lucanians (Leroy et al., 2013). The modern word "salami", which refers to a fermented sausage, probably originates during the Middle Age from the vulgar Latin "*salamen*" that derives from the word "salt". Between the 12th and the 17th centuries, a plenty of charcuterie products, mainly based on the fermentation of swine meat, were developed in Central Italy where the pork butchers from Norcia village (Central Italy) gave birth to the term "*norcino*" (Aquilanti et al., 2012). Confirming the loyalty to the oldest tradition of pork butchery, in the Marche Region a wide range of culinary specialties based on the fermentation of pork meat is still produced; among these, *Ciauscolo* salami represents an undisputed food delicacy with unique sensory traits.

The name *Ciauscolo*, also referred to as *Ciavuscolo*, originates from the words "*ciabusculum*" or "*cibusculum*" that in Latin were used to describe a small piece of food or snack which peasants would eat in small amounts during breaks and between main mealtimes. *Ciauscolo* salami obtained the Protected Geographical Indication (PGI) status, in accordance with Commission Regulation (EC) No 729/2009 of 10 August 2009. According to the production disciplinary, this salami can be produced in a well-defined geographical area covered by four (Ancona, Ascoli Piceno, Fermo, and Macerata) out of the five provinces of the Marche region (central Italy). The cuts of pork (e.g. Italian Large White, Italian Landrace, Italian Duroc breeds) that can be used in the meat batter include bacon, shoulder, ham and loin trimmings. The following ingredients are also added at different concentrations: salt, grinded black pepper, wine, and crushed garlic. The addition of lactose, dextrose, fructose and sucrose is allowed. Among the additives with preservative and antioxidant functions, the use of L-ascorbic acid (E300), sodium ascorbate (E301), potassium nitrate (E252) is also permitted in accordance with the maximum limits established by Regulation (CE) 1129/2011. Finally, the use of milk flours, caseinates and other coloring compounds is expressly prohibited.

The chilled meat is minced using a 2–3 mm plate and then mixed with lard, salt, grinded black pepper, wine, crushed garlic, and eventually added additives. The mixture is then stuffed into bovine or pork-intestine casings and ripened for at least 15 days. The end product is characterized by 15% protein and a fat content comprised between 32% and 42%. *Ciauscolo* salami, which length ranges from 15 to 45 cm and weight ranges between 400 and 2500 g, is characterized by a soft, homogeneous and rosy paste with high ductility for spreading. The taste is low acidic and savoury, and the flavour is spicy and aromatic (Aquilanti et al., 2007).

To the best of the authors' knowledge, so far, only a few studies have investigated the microbiota of *Ciauscolo* salami by applying conventional culture-dependent and –independent techniques, such as Amplified Ribosomal DNA Restriction Analysis (ARDRA) of the isolated cultures (Federici et al., 2014) and Polymerase Chain Reaction-

Denaturing Gradient Gel Electrophoresis (PCR-DGGE) analysis of the DNA extracted directly from either the salami samples (Silvestri et al., 2007) or bulk of cells harvested from countable plates (Aquilanti et al., 2007). Due to the acknowledged limitations of these methods, only the main components of *Ciauscolo* salami microbial community were revealed, with a narrow pool of species successfully identified. Moreover, no studies focused on the microbiota-associated volatile profile of this dry cured meat specialty have been carried out, yet.

It is noteworthy that the current availability of high throughput sequencing methods allows direct sequencing of the rRNA genes of mixtures of barcoded food samples thus increasing the amount of information obtained from complex food matrices (Cocolin and Ercolini, 2015). In line with this recent evolution of investigations into the microbial diversity of food ecosystems, a polyphasic approach based on viable counting, amplicon-based sequencing (Illumina sequencing) and PCR real-time was applied to disclose the still undetected biodiversity of *Ciauscolo* salami. To this end, an artisan manufacture (used as model) produced in the place of origin according with the PGI production disciplinary without any starter cultures and preservatives was analyzed during its natural fermentation. The characterization of small volatile organic compounds (VOCs) was also carried out to shade light on the sensory profile of this cured meat specialty.

2. Materials and methods

2.1. Model Ciauscolo salami production

Twenty artisan and industrial *Ciauscolo* producers located in the geographical area indicated by the PGI disciplinary were interviewed about key parameters referred to products (weight, diameter, and length) and production processes (meat cuts used, % of fat, additional ingredients, sugars, use of starter cultures, use of nitrate/nitrite, fermentation and ripening conditions). Based on data overall collected, an artisan plant located in the Macerata province was selected among those not using either starter cultures or nitrate/nitrite for sampling of a model *Ciauscolo* salami manufacture representing an excellence of this dry cured meat sausage from the Marche Region. The collection of data from local *Ciauscolo* manufacturers was supported by the *Agenzia per i Servizi nel Settore Agroalimentare delle Marche* (ASSAM) within a funded Project aimed at valorizing local high-value food products.

Two independent manufacturing trials (marked as batch 1 and batch 2) were performed. Both the trials were realized by an artisan producer located in the geographical area of production (Macerata province of the Marche Region), in accordance with the *Ciauscolo* production disciplinary. The first batch was produced in February, whereas the second batch was produced in May. According to the disciplinary for the production of *Ciauscolo* salami, meat batter was

prepared using the following swine cuts: shoulder 32%, belly (including fat) 40%, loin 15% and ham 13%. Moreover, salt 2.7 %, ground black pepper 0.4%, ground garlic 0.1% and white wine (10 mL/kg) were also added; no nitrates and no starter cultures were used. For each batch a total of 40 kg of meat batter was processed. The meat batter was minced twice with a 3 mm plate and stuffed into bovine slim bowels previously washed in a mixture of water and white wine vinegar 50% (v/v). In accordance with the disciplinary for the production of *Ciauscolo* salami, the drying was performed under controlled conditions, at a relative humidity (R.H.) of 85% for 6 days at 4 °C. Moreover, the ripening was carried out at 82% R.H for 20 days at 10 °C. For each batch, *Ciauscolo* salami approximately weighing 600 g, with 28 cm length and 6 cm width, were produced (Fig. 1). Sampling was performed at 0, 5, 10, 20 days; for each ripening time and each batch, three fermented sausages (for a total of 24 sausages) were collected. Samples were aseptically collected using sterile instruments and sterile bags (Nasco Whirl-Pak Easy-To-Close Bags, Fisher Scientific Italia, Rodano, Italy). The samples were rapidly refrigerated and analysed on the same day of collection.

2.2. Physical-chemical analyses

pH values of the model *Ciauscolo* salami samples were measured with a pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy) directly inserted in the food matrix. For each sample, pH measurements, performed at the core of the sausages, were carried out in duplicate and the results reported as mean ± standard deviation.

For total titratable acidity (TTA) assessment, 10 g of each sample was blended in 90 mL distilled water and the suspensions were then titrated with 0.1 N NaOH and the results expressed as mL of NaOH (Pramualkijja et al., 2016). TTA analyses were carried out in duplicate and the results reported as mean ± standard deviation.

The acetic acid and lactic acid concentrations were measured using the Acetic Acid (Acetate Kinase Manual Format) test kit and D-/L-Lactic Acid (D-/L-Lactate) (Rapid) test kit, respectively, from Megazyme (Bray, Ireland).

The water activity (a_w) was assessed in accordance with the ISO 21807:2004 standard method using an Aqualab 4TE apparatus (Meter Group, Pullman, USA).

Lipid oxidation was monitored by determining the peroxide value (PV, mEq peroxide kg sample^{-1}) according to AOAC method 965.33 (1990). Duplicate PV determinations were performed, and results reported as mean ± standard deviation.

2.3. Microbial counts

Prior to microbiological analysis, sausage casing was removed under sterile conditions using a sterile scalpel, then, 10 g aliquots of each analyzed sausage were added with 90 mL sterile water containing 1 g/L bacteriological peptone. The suspension was homogenized in Stomacher apparatus (400 Circulator, International PBI, Milan, Italy) for 2 min at 230 rpm. Ten-fold serial dilutions were prepared for the viable counts of the following groups of microorganisms: i) lactic acid bacteria enumerated on MRS agar (VWR Prolabo Chemicals, Leuven, Belgium) (De Man et al., 1960) with incubation at 37°C for 48-72 h; ii) coagulase negative cocci counted on mannitol salt agar (MSA) (VWR Prolabo Chemicals) (Chapman, 1945; Bannerman, 2003) with incubation at 37°C for 24-48 h; iii) *Enterobacteriaceae* enumerated on violet red bile glucose agar (VRBGA) (VWR Prolabo Chemicals) (American Public Health Association, 1978) incubated at 37°C for 24 h and iv) yeasts and molds counted on Rose Bengal Chloramphenicol Agar (VWR Prolabo Chemicals) (Baggerman, 1981) with incubation at 25°C for 72 h. Cycloheximide (250 mg/L) was added to MRS agar to prevent the growth of eumycetes.

The results of viable counts, expressed as the Log of colony-forming units (cfu) per gram of sample, were reported as mean value of the two biological and three technical replicates ± standard deviation.

Finally, a miniVIDAS apparatus (bioMérieux, Marcy l'Etoile, France) was used to assess the presence/absence of *Listeria monocytogenes* and *Salmonella* spp. through the enzyme-linked fluorescent assay (ELFA) method, in accordance with the AFNOR BIO 12/11-03/04 and AFNOR BIO 12/16-09/05 standard methods, respectively (Haouet et al., 2017). *L. monocytogenes* detection was carried out through pre- enrichment in half- Fraser broth (VWR Prolabo Chemicals) with incubation for 24 to 26 hours at 30°C ± 1°C, followed by enrichment in Fraser broth (VWR Prolabo Chemicals) with incubation for 24 hours at 37°C ± 1°C. *Salmonella* spp. detection was carried out through pre-enrichment in Buffered Peptone Water (VWR Prolabo Chemicals) with incubation for 16 to 22 hours at 37°C ± 1°C, followed by enrichment in SX 2 broth (bioMérieux) with incubation for 24 hours at 42°C ± 1°C.

2.4. RNA extraction and cDNA synthesis

1.5 mL of each sample homogenate (dilution 10^{-1}) prepared as described above was centrifuged for 10 min at 16000 g; the cell pellets were then protected with RNA later Stabilization Solution (Ambion, Foster City, CA, USA) and stored at -80°C until use. The total microbial RNA was extracted from the cell pellets using E.Z.N.A. Bacterial RNA Kit (Omega Bio-tek, Norcross, GA, USA) in accordance with manufacturer's instructions. The extracted RNAs was checked for quantity, purity and integrity as described by Garofalo et al. (2017). Extracted RNAs were also checked for the presence of residual DNA by PCR amplification with universal prokaryotic primers 27f and 1495r (Weisburg et al.,

1991) which resulted negative. SensiFAST cDNA Synthesis Kit for RT-qPCR (Bioline, London, UK) was used for cDNA synthesis in accordance with manufacturer's instructions.

2.5. Amplicon target sequencing

cDNA was used as template in order to amplify the V3-V4 region of the 16S rRNA (Klindworth et al. 2013) as well as D1, D2 domain of the 26S (Mota Gutierretz et al. 2018), following procedure and primers already reported. PCR template were then purified and processed following the Illumina metagenomic pipeline. Sequencing was performed in a MiSeq instrument in a 2X250bp configuration. After sequencing, reads were analyzed through QIIME v. 1.9 (Caporaso et al. 2011). For 16S, data were analyzed following the pipeline described by Ferrocino et al (2017). OTU clustering was obtained at 99% of similarity and the greengenes database was used for taxonomic assignment. For 26S data, the pipeline by Mota Gutierretz et al. 2018 was used and the 26S databases from the same authors was used for assessing the taxonomy. Taxonomy assignment for 16S and 26S was double checked on BLAST suite tools.

2.5.2. Statistical analysis

OTU (for 16S and 26S) were rarefied at the lowest number of sequence/sample for each datasets, and the OTUs table display the highest taxonomic resolution reached. Alpha diversity index was calculated by the vegan function of R. OTUs table and diversity index were then imported in R to perform statistical test in order to find difference in the sample as a function of the batch or the ripening time. Non parametric spearman correlation analysis was performed on OTUs or volatilome data through the *psyc* function of R and visualized by the *corrplot* function of R. PICRUSt (Langille et al., 2013) was used to predict the abundances of gene families based on 16S rRNA sequence data and data were visualized by the function heatplot of the made4 package of R.

2.6. Real-time PCR analyses for the detection of botulinic toxins genes

Clostridium botulinum was analyzed in accordance with the multiplex real-time PCR method of the Italian National Reference Centre for botulism (http://old.iss.it/binary/cnrb/cont/CNRB31.010.pdf) for the detection of the target genes *bont/A, bont/B, bont/E, bont/F,* and *4gyrB (CP*) that codify for botulinic toxins.

Briefly, 25 g of sample were blended in 225 mL Triptone Peptone Glucose Yeast extract (Microbiol Diagnostici, Italia), incubated in anaerobiosis at 30°C for 96 h and then extracted with 6% Chelex-100 (Biorad, Milan, Italy). The

amplification was carried out using the Kit QuantiTect multiplex No Rox (Qiagen) in the Stratagene Mx3005P (Agilent Technologies) thermal cycler and the primers and probes nucleotide sequences listed in Supplementary Table 1. Two different Master mixes were used concurrently, with the following thermal profile: 1 cycle at 95°C for15 min followed by 40 cycles at 94°C for 30 s and 56°C for 90 s.

Real-time PCR analyses were performed on a RotorGene Q thermal cycler (Qiagen, Hiden, Germany) exploiting TaqMan chemistry. All target probes employed were dual-labeled with 5'-FAM and a 3'-nonfluorescent quencher (as specified below). The oligonucleotides were purchased from ThermoFisher Scientific (Milan, Italy) and from LCG Biosearch Technologies (Petaluma, CA, USA). The reaction mixtures were all prepared at a final 25 µl reaction volume. Molecular-grade H_2O was included in each analytical session as a negative control, as well as DNA from reference strains as positive controls. Fluorescence was measured in the green channel for the target genes, and in the yellow channel for the Internal Amplification Control.

2.7. GC-MS analysis of volatile components

Two grams of fresh salami were weighed in a 10 mL screw cap septum vial. The static headspace was sampled by a 65 µm PDMS/DVB SPME fibre (Supelco, Bellefonte, PA) according to Savini et al. (2017). Chromatographic separation of volatiles was performed by a fused silica capillary column ZB-5 (30 m L, 0.25 mm ID, 0.25 µm FT; Phenomenex, Torrance, CA) mounted on a Varian 3900 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA); the injector was operating in splitless mode for 0.1 min at a constant temperature of 250 °C; oven temperature was increased from 40 °C to 220 °C at a rate of 6 °C/min, then held at the final temperature for 5 min; carrier gas (He) was set at constant flow mode (1.0 mL/min). The gas chromatograph was coupled to an ion trap mass detector Saturn 2100T (Varian Analytical Instruments, Walnut Creek, CA): the trap and the transfer line were set at 200 °C and 220 °C, respectively; electron impact (70 eV) mass spectra were acquired in the mass range of 31-250 amu.

Volatile compounds were identified by matching mass spectral data collected in the NIST/EPA/NIH Mass Spectral Library (Version 2.0a, build July 1 2002; National Institute of Standards and Technology) and Kovats Retention Indexes (RIs) available in the public access database Pubchem (https://pubchem.ncbi.nlm.nih.gov/). A C8-C20 normal alkanes mixture (Sigma-Aldrich, St. Louis, MO) was used to calculate RIs. An automated spreadsheet (Lucero et al., 2009) was used for simplifying the calculation of RIs ok unknown components and speeding up the comparison with published indexes.

2.8. Data analysis

Principal component analysis (PCA) was carried out on autoscaled data matrix to explore relationships among experimental variables (volatile amounts) and to detect possible clusters of samples. One-way ANOVA was used to analyse the effect of ripening time on the dependent variables (aroma components), separately for each batch: the Tukey-Kramer's Honest Significant Difference (HSD) test was used and the level of significance was set to 0.05. All statistical analyses were carried out by the software JMP® Version 10 (SAS Institute Inc., Cary, NC).

3. Results

3.1 Physical-chemical characterization

The results of physical-chemical characterization of the analyzed raw material *Ciauscolo* salami are reported in Table 1. In more detail, pH values ranged from 5.87±0.00 to 5.86±0.00 at t0 in batch 1 and batch 2, respectively. pH values progressively decreased along ripening and, at t20, values comprised between 5.49±0.01 and 5.69±0.01 in batch 1 and batch 2, respectively, were recorded. In both the analyzed batches, pH values at t20 were significantly lower than those recorded at t0.

Water activity values (a_w) at t0 ranged from 0.959±0.003 to 0.960±0.002 in batch 1 and batch 2, respectively. At t20, a_w values recorded in both the analyzed batches were 0.954.

Regarding TTA (expressed as mL of 0.1N NaOH), a progressive increase was observed along ripening. At t0, values comprised between 6.1±0.14 and 5.2±0.2 in batch 1 and batch 2, respectively, were detected; whereas at t20, TTA values ranged from 9.2±0.57 to 7.0±0.9 in batch 1 and batch 2, respectively. In both the analyzed batches, TTA values at t20 were significantly higher than those recorded at t0.

As for organic acids, both lactic and acetic acids showed higher values at t20 in respects with those recorded at t0. In more detail, lactic acid values at t0 ranged from 0.414±0.075 to 0.445 ±0.050 g/100 g in batch 1 and batch 2, respectively; whereas, at t20, values from 0.696±0.090 to 0.614 ±0.047 g/100 g in batch 1 and batch 2, respectively, were detected. Acetic acid values at t0 were comprised between 0.008±0.001 and 0.008 ±0.005 g/100 g in batch 1 and batch 2, respectively; whereas, at t20 acetic acid concentration ranged from 0.055±0.010 to 0.059 ±0.005 g/100 g in batch 1 and batch 2, respectively.

Finally, undetectable lipid oxidation, expressed as mEq peroxide kg sample^{-1}, was observed in all the analyzed samples.

3.2 Microbiological analyses

The results of viable counts for the enumeration of bacteria and eumycetes are reported in Table 2. In both the analyzed batches of model *Ciauscolo* salami, lactic acid bacteria counts showed a progressive increase from t0 to t20, attesting at the highest values at the end of ripening (t20). Average values at t20 were comprised between 8.18±0.06 and 7.55±0.03 cfu/g in batch 1 and batch 2, respectively.

Coagulase negative cocci showed variable trends among batches with the lowest values detected at t0 in batch 1 and 2, as well as at t20 and t10 in batch 1 and 2, respectively.

Enterobacteriaceae showed a progressive decrease from t0 to t20. In more detail, average values at t0 were comprised between 3.05±0.08 and 1.93±0.09 cfu/g in batch 1 and batch 2, respectively; whereas, at t20, counts between 2.22±0.14 and 1.42±0.17 cfu/g in batch 1 and batch 2, respectively, were detected.

In both the two analyzed batches, yeasts showed the lowest average values at t0 with counts comprised between 3.97±0.24 and 3.81±0.06 cfu/g in batch 1 and batch 2, respectively. A progressive increase of yeasts was generally observed until the end of ripening.

Molds showed viable counts comprised between 2.00±0.00 and < 2.00 cfu/g in samples from batch 1 and always < 2.00 cfu/g in samples from batch 2.

No samples revealed the presence of *L. monocytogenes* or *Salmonella* spp. in 25 g of product, irrespective of the sampling time.

Finally, multiplex real-time PCR revealed the absence of the target genes *bont/A, bont/B, bont/E, bont/F,* and *4gyrB (CP*), encoding botulinic toxins, in all the samples analyzed.

3.3. 16S rRNA gene amplicon target sequencing

The total number of sequences obtained from sausages reached 292.044 raw reads. After quality filtering, a total of 197186 reads were used, with an average value of 24.648 reads/sample, and a mean sequence length of 465 bp. The Estimate sample coverage showed a satisfactory coverage for all samples (> 98%) while alpha diversity index did not showed significant difference as a function of the ripening. By principal component analysis a development in the microbiota composition was observed (Adonis, P=0.012) as a function of the ripening time (data not shown).

Taking into the account the microbiota composition at the highest taxonomic level (Figure 2), we observed a predominance of *Pseudomonas fragi* (33% and 9% of the relative abundance at t0 and t5, respectively, in batch 1, and 30% and 44% at t0 and t5, respectively, in batch 2), *Campylobacter* (4% and 76% of the relative abundance at t0 and t5, respectively, in batch 1, and 46% and 4% at t0 and t5, respectively, in batch 2) and *Psychrobacter* (10% and 0.8% of the

relative abundance at t0 and t5, respectively, in batch 1, and 2% and 3% at t0 and t5, respectively, in batch 2) in the first 5 days of ripening. *Brochothrix thermosphacta* was detected at highest percentage at 5 and 10 days of ripening only in batch 2 (24% and 77% of the relative abundance at t5 and t10, respectively), while staying below 3% in batch 1. *Lactobacillus sakei* was observed predominant in batch 1 at the end of ripening (16% and 10% of the relative abundance after 10 and 20 days of ripening, respectively), whereas was predominant in batch 2 at the beginning of the ripening (at about 8%).

Lactobacillus algidus and *Leuconostoc carnosum* dominated the microbiota at the end of the ripening. In more detail, *Lactobacillus algidus* was detected at 45% and 50% of the relative abundance at t10 and t20, respectively, in batch 1, and at 8% and 49% at t10 and t20, respectively, in batch 2. *L. carnosum* was detected at 29% and 31% of the relative abundance at t10 and t20, respectively, in batch 1, and at 2% and 41% at t10 and t20, respectively, in batch 2 (Figure 2).

3.4. Mycobiota composition

The total number of sequences obtained from sausages reached 417.976 raw reads. After quality filtering, a total of 371.731 reads were used, with an average value of 30.963 reads/sample, and a mean sequence length of 380 bp. The Estimate sample coverage showed a satisfactory coverage for all samples (> 98%) but no difference were observed for the other diversity index as a function of the ripening or the batch. By principal component analysis, a development in the mycobiota composition was observed (Adonis, P=0.012) as a function of the ripening time (data not shown). The core mycobiota of the sausages (Figure 3) was characterized by the predominance of *Aureobasidium* in all the samples (relative abundance ranging from 1% to 9%), *Cladosporium cladosporioides* (ranging from 1% to 25% with the maximum after 5 days in samples from batch 1); *Debaryomyces hansenii* that was found to be dominant at 10 days in batch 1 while remaining higher up to 50% in batch 2 from 10 days till the end of ripening. *Glomus hyderabadensis* was observed in batch 1 higher of 15% from the fifth day till the end while in batch 2 was predominant at the beginning of the ripening (about 34% of the relative abundance in the first five days of ripening). *Tilletiopsis washingtonensis* (30%) was observed only in meat batter of batch 1, whereas *Kurtzmaniella zeylanoides* (41%) was predominant at the end of ripening only in batch 1 (Figure 3).

3.5. Correlation analysis

By plotting the correlation between microbiota and mycobiota composition of the sausages (Figure 4), the presence of *Campylobacter* was associated with the presence of *Aspergillus* and *Malassezia yamatoensis*, *Lactobacillus algidus*

with *Kurtzmaniella* and *Penicillium roqueforti*, while co-excluding *Aspergillus*. *P. fragi* showed the highest co-exclusion pattern with most of the mycobiota including those with members of *Kurtzmaniella* and *Penicillium* (Figure 4).

3.6. Inferred metagenomic analysis

To study the possible metabolic pathways of sausages microbiota PICRUSt was used. In more detail, an enrichment of several metabolic pathways involved in carbohydrates metabolism (KEGG genes) belonging to glycolysis, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, galactose metabolism was observed in batch 1. On the other hand, samples from batch 2 showed the predominance of KEGG genes involved in fatty acid metabolism, ether lipid metabolism, biosyntesis of unsaturated fatty acids, propanoate metabolism, valine leucine and isoleucine metabolism (Figure 5).

3.7. GC-MS analysis of volatile components

Volatile compounds detected in the static headspace of the two batches of salami samples are reported in Table 3. In more detail, a total of 53 volatile substances were fully or tentatively identified.

The volatile profiles were dominated by the spices derived components (black pepper and garlic cloves), throughout the whole ripening time. Limonene, sabinene, α- and β-pinene, 3-carene, and α-thujene were the most represented monoterpene hydrocarbons, whereas β- and α-copaene were the most represented sesquiterpene hydrocarbons. Allyl methyl sulphide and diallyl disulphide were the major aliphatic sulphur compounds, together with diallyl sulphide and allyl methyl disulphide. The presence of hexanal was also detected.

Samples collected from batch 1 were characterized by higher levels of aliphatic aldehydes (hexanal, heptanal, octanal) than those collected from batch 2. Despite the same total amount of sulphur compounds, allyl methyl sulphide was more represented in samples from batch 1, whereas disulphides (allyl methyl and diallyl) were more represented in batch 2 samples. Higher levels of monoterpene hydrocarbons (α-thujene, α-pinene, camphene, sabinene, β-pinene, myrcene, α-terpinene, limonene, γ-terpinene, terpinolene) were detected in the batch 2 samples, whereas average levels of sesquiterpene hydrocarbons did not show significant differences between fermented sausages collected in both the two batches.

Regarding the effect of ripening time on aroma composition, different behaviours have been observed between batches. In more detail, a decrease of methyl butanols and a strong increase in ethyl isopentanoate level were observed in batch 1

samples, whereas batch 2 samples were characterized by very limited changes in aroma composition during ripening where only an increase of ethyl ester levels (ethyl isopentanoate) was observed.

4. Discussion

Fermented sausages are obtained through complex biochemical and microbiological activities that allow the raw meat to be transformed into a safe product characterized by pleasant sensory traits. Acidification of the meat batter undoubtedly represents the first step in the evolution of the microbial metabolism during fermentation of sausages. Regarding pH measured in the analyzed model *Ciauscolo* salami samples, the recorded values were higher than those already reported by both Aquilanti et al. (2007) and Trani et al. (2010) for the same product. The emerged differences might be ascribed to the highest initial pH value and glucose concentration of raw meat used for production of the fermented sausages that, as reported by Bover-Cid et al. (2008), can affect the degree of acidification. In the samples under study, the decrease in pH was associated with an increase in TTA values, according with the ripening time, thus, attesting the accumulation of microbial-derived organic acids in the fermented meat. Organic acid values increased according to ripening time, as well; among the detected organic acids, lactic acid was the most abundant, whereas small quantities of acetic acid were detected, thus suggesting a minor role played by hetero-fermentative lactic acid bacteria in respect with homofermentative species. In dry fermented sausages, acetic acid is undesirable as it can reduce the palatability and the overall quality of the product. As already reported by Aquilanti et al. (2007), the early drop in pH is a distinctive trait of this short-ripened salami, though differences between the two batches were seen, likely as a consequence of the intrinsic variability of artisan manufacturing. Notwithstanding, the final pH values reached by the analyzed model *Ciauscolo* salami were in accordance with those foreseen by the production disciplinary.

As for a_w, the values recorded in the present study were higher than those reported by Aquilanti et al. (2007) and Trani et al. (2010) in the same salami at the end of its ripening (t20), ranging from 0.87±0.01 to 0.93±0.01 and from 0.88±0.01 to 0.91±0.01, respectively.

In the present study, the counts of lactic acid bacteria progressively increased from t0 to t20 reaching values that were in accordance with those previously detected by Aquilanti et al. (2007), Silvestri et al. (2007) and Trani et al. (2010). The values were also in accordance with those foreseen by the production disciplinary of *Ciauscolo*. It is noteworthy that, the release/degradation of free amino acids due to lactic acid bacteria activity strongly affects the presence of non-volatile and volatile compounds in the final product. Moreover, these pro-technological bacteria are known to prevent the oxidation of unsaturated free fatty acids (Trani et al., 2010). This trait combined with the short ripening time of *Ciasculo* might explain the undetectable lipid oxidation observed in the samples analyzed in the present study.

Stable counts of coagulase-negative staphylococci were observed throughout ripening in all the samples. These counts were notable lower than those reported by Aquilanti et al. (2007), Silvestri et al. (2007) and Trani et al. (2010) for the same microbial group.

Regarding *Enterobacteriaceae*, a progressive reduction in viable counts was seen from t0 to t20, although with differences among batches likely due to the initial level of contamination. These counts were slightly higher than those reported by Aquilanti et al. (2007) for *Ciauscolo* salami analyzed at the same sampling times. However, Aquilanti et al. (2007) reported lower pH values in respect with those recorded in the present study, thus likely explaining the weak *Enterobacteriaceae* highlighted in the present study. The persistence of *Enterobacteriaceae* in the analyzed *Ciauscolo* samples is consistent with the data reported by Castaño et al. (2002) for *Chorizo de cebolla* salami produced without either nitrate/nitrite or starter cultures. Members of the family *Enterobacteriaceae* show different resistances to low pH values. Indeed, as reported by Dinardo et al. (2019), lactate and acetate can inhibit *Enterobacteriaceae* species (e.g. *Cronobacter sakazakii*, *Klebsiella pneumoniae*, etc.) starting from pH 5.

Viable counting allowed detecting a slight increase of yeasts from t0 to t20 in both the analyzed batches. This yeast evolution is in accordance with yeast counts assessed at the same ripening times by Aquilanti et al. (2007) or at the end of ripening by Silvestri et al. (2007) and Trani et al. (2010). In fermented sausages, yeasts are protective microorganisms against oxidation and allow a proper drying by protecting the sausage against fluctuation in humidity (Lücke, 2000). These microorganisms also utilize lactic acid produced by lactic acid bacteria and release peptides, free amino acids and free fatty acids through proteolysis and lipolysis, thus contributing to flavor formation (Flores et al., 2015). Even pro-technological molds (e.g. *Penicillium* species) can be highly desired in some dry-cured meat, where they penetrate deeply with their mycelia, contributing in both flavor formation and protection against oxidation and detrimental microorganisms (Magistà et al., 2017). The low levels of molds detected in the samples analyzed in the present study are likely due to the short ripening time of the analyzed *Ciauscolo* salami manufacture.

As for *C. botulinum*, the absence in all the analyzed samples of the target genes *bont/A*, *bont/B*, *bont/E*, *bont/F*, and *4gyrB (CP)*, encoding botulinic toxins, revealed no risks associated with the presence of this pathogen. The absence of *C. botulinum*, as well as that of *Salmonella* spp. and *L. monocytogenes* in all the analyzed samples reflected the high quality of the raw materials and the good hygiene conditions applied during manufacturing.

Although the use of rRNA as an indicator of the metabolic state in microbial assemblages can present some limitations (Blazewicz et al., 2013), the analysis of this nucleic acid is still widely applied to evaluate the metabolically active microbial communities in food matrices (Garofalo et al., 2017). The application of amplicon sequencing to microbial rRNA, directly extracted from the model *Ciauscolo* samples, allowed previously undiscovered major and minor taxa in *Ciauscolo* to de detected.

Regarding the presence of *P. fragi*, as part of the core microbiota in the meat batter, this bacterial species has already been detected by Cardinali et al. (2018) and Greppi et al. (2015) in the meat batter used in the production of Fabriano-like fermented sausages and traditional *Piemontese* sausages, respectively. It is noteworthy that, as reported by Ercolini et al. (2010), meat represents an ecological niche for *P. fragi* where such a spoilage microorganism with proteolytic and lipolytic capacities can release volatile molecules (e.g. esters conferring fruity odors to the meat). As reported by Lebert et al. (1998), among members of the genus *Pseudomonas* naturally contaminating meat, a succession of species is generally observed during meat processing. In such a context, *Pseudomonas fluorescens* usually dominates at the beginning of the slaughtering and *P. fragi* becomes dominant during the chilling process of meat.

Regarding *B. thermosphacta*, this Gram-positive, non-spore-forming, non-motile, homofermentative, facultative and anaerobic bacterial species has already been detected by different authors in meat batters used for production of fermented sausages as *Fabriano* sausage (Cardinali et al., 2018), *Piemontese* sausage (Greppi et al., 2015) as well as in casings used for production of *Salame Mantovano* (Pisacane et al., 2015). As reported by Casaburi et al. (2014), pork meat was the first source of isolation of *B. thermosphacta*, thus confirming a strong adaptation of this spoilage microorganism to meat environment. Indeed, when growing in meat, *B. thermosphacta* uses glucose as a preferred substrate and can produce off-flavors including a pungent 'cheesy' odor (McClure et al., 1993).

As reported by Greer and Dilts (1995), *P. fragi* and *B. thermosphacta* are strongly inhibited by the presence of lactic acid. Indeed, as the lactic acid content and counts of pro-technological microorganisms (e.g. lactic acid bacteria) increased along ripening, a progressive decrease in the analyzed samples of *P. fragi* was observed from t5 to t10. Moreover, being *P. fragi* obligate aerobe, its reduction was also favored by the progressive decrease of oxygen in the meat batter due to the metabolic activities of pro-technological microorganisms (Wang et al., 2018). *B. thermosphacta* could be detected until t10, likely due to its ability to grow in meat even without oxygen (Casaburi et al., 2014).

Campylobacter and *Psychrobacter* were also detected in the meat batters of both the analyzed batches. The genus *Campylobacter* encompasses well-known enteric human pathogens as *Campylobacter jejuni*. Its presence in the food environment is often associated with poultry meat, although species of thermotolerant *Campylobacter* have also been detected in bovine or swine meat (Petruzzelli et al., 2014; Osimani et al., 2017). It is noteworthy that, at the end of ripening (T20), *Campylobacter* was never detected.

Even *Psychrobacter*, a bacterial genus encompassing halotolerant, psychotropic and oxidative bacteria, has already been detected with low abundance in meat batter of other different fermented sausages like *Fabriano* salami (Cardinali et al., 2018) and Chinese dry- and smoked-cured sausage as well as in wild boar and deer meat sausages (Wang et al., 2018; Mrkonjic Fuka, et al. 2019).

At the end of ripening (t20), a few bacterial species were dominating the microbiota of *Ciauscolo* salami where *L. algidus* was prevailing in both the analyzed batches. *L. algidus* has rarely been recovered from fresh meat and it represents a meat spoilage bacterium that often dominates the bacterial communities of chilled packaged meat (Säde et al., 2020). It is a cold-adapted lactic acid bacterium that, in meat, is often associated with the production of "sour and intense" or "butyric" off-odors (Säde et al., 2020). To the authors' knowledge, *L. algidus* has never been detected before in *Ciauscolo* salami, although this species has already been found by other authors in traditional fermented sausages produced in Northern Italy (Cocolin et al., 2009; Greppi et al., 2015) and Belgium (Janssens et al., 2012). More recently, *L. algidus* has also been detected by Bouju-Albert et al. (2018) among the most prevalent species in French fresh pork sausages and by Quijada et al. (2018) in *Chorizo de León*, although in this latter product as a minority species. However, the contribution of *L. algidus* in the sensory quality of fermented meat is still unknown (Säde et al., 2020). Interestingly, Mansur et al. (2019) found that, in raw beef cuts, *L. algidus* was significantly positively correlated with the presence of acetic and/or butanoic acid, and that it was negatively correlated with the presence of alcohols and esters. Mansur et al. (2019) also suggested a potential protective role of *L. algidus* towards spoilage-bacterial growth, thus reducing the presence of several spoilage-related volatile compounds.

Among the detected *Leuconostoc* species, *L. carnosum* represented a majority OTU in both the batches of *Ciauscolo* salami. Although *Leuconostoc* species have already been detected in *Ciauscolo* salami (Federici et al., 2014), to the authors' knowledge this is the first report of *L. carnosum* in this specialty product. This species has previously been isolated from *Levačka* sausage, a fermented dry sausage produced in central Serbia (Borovic et al., 2015). Interestingly, the manufacturing process of *Levačka* was approximately the same duration of that of *Ciauscolo* salami, attesting at about 20 days. In *Levačka*, *L. carnosum* was detected as a minority species during the early stage of ripening (Borovic et al., 2015). The same species has also been isolated in other traditional Italian fermented sausages (Bonomo et al., 2008; Greppi et al., 2015).

As for the occurrence of *L. sakei*, detected in both the batches with different relative abundances, this species has constantly been detected in *Ciauscolo* salami, irrespective of the detection technique used. Indeed, in this dry cured meat product, *L. sakei* was detected as the major lactic acid bacteria species using both culture-independent (Silvestri et al., 2007; Aquilanti et al., 2007) and -dependent analytical approaches (Federici et al., 2015). The dominance of *L. sakei* in *Ciauscolo* salami is in accordance with previous findings on the occurrence of this species in numerous Italian, (e.g. *Fabriano*, *Soppressa*, *Salame Mantovano*, *Salame Cremonese*, *Salame Bergamasco*, *Salame Piacentino*, *Salame Napoli*, *Soppressata del Vallo di Diano*, *Salame di Senise*, *Salsiccia Sarda*, *Salsiccia* from *Suino Nero dei Nebrodi*, etc.) and European (e.g. *Chorizo*, *Salsichòn*, *Fuet*, *Androlla*, *Alherira*, etc.) fermented sausages, where it was almost ubiquitous due to a high adaptability to the peculiar temperature and a_w conditions adopted for manufacturing and

fermentation (Aquilanti et al., 2016; Cardinali et al., 2018; Janssens et al., 2012). As far as the role of *L. sakei* in meat-based products is concerned, strains ascribed to this species are known to express proteinases and aminopeptidases catalyzing the hydrolysis of proteins of sarcoplasmic and myofibrillar origin (Fadda et al., 1999).

Metataxonomic sequencing also allowed the mycobiota of model *Ciauscolo* salami to be disclosed.

Regarding the core mycobiota, as far as the authors know, this is the first report of *Aureobasidium* in *Ciauscolo* salami. This genus has previously been reported as one of the causative agents of black spot in fermented sausages (Lozano-Ojalvo et al., 2015; Papagianni et al., 2007). The presence of this yeast-like fungi in food is generally of environmental origin (Osimani et al., 2016). Regarding *Ciauscolo* salami, additional ingredients, like herbs and spices (e.g. powdered pepper and garlic) added to the meat batter could have contributed to the contamination with *Aureobasidium*. Interestingly, Sørensen et al. (2008) reported the isolation of *Aureobasidium* from the environment of a Danish meat processing plant for production of fermented sausages, thus suggesting the adaptation of this genus to this peculiar environment.

C. cladosporioides, detected in all the analyzed model *Ciauscolo* salami samples, has previously been found in South America dry fermented sausages as either a predominant (Parussolo et al., 2019) or minority (Canel et al., 2013) species. Fungi belonging to the genus *Cladosporium* are considered as GRAS (*Generaly Regarded as Safe*) and, since they can inhibit toxigenic fungi, their use as biological control agents has so far been proposed. Moreover, *C. cladosporioides* possesses the competitiveness to germinate and develop in meat, thus delaying the germination of toxigenic fungi like *Aspergillus ochraceus*, *Aspergillus niger* and *Fusarium verticillioides* (Parussolo et al., 2019).

As for *D. hansenii*, this lipolytic foodborne yeast has already been detected in both *Ciauscolo* (Silvestri et al., 2007; Aquilanti et al., 2007) and other Italian (Baruzzi et al., 2006; Gardini et al., 2001; Giarratana et al., 2014; Murgia et al., 2019) salami as a prevalent component of the fungal community. *D. hansenii* is known to hydrolyze pork muscle sarcoplasmic proteins, thus contributing to the definition of the aroma and, hence, to the sensory traits of fermented sausages. It therefore represents a key microorganism in the production of fermented sausages since its metabolic activity leads to the increase of ammonia concentration and the decrease of lactic acid and acetic acid content, thus leading to a slight pH increase (Olesen and Stahnke, 2000). In recent years, the use of *D. hansenii* has been proposed as a potential tool to control toxigenic penicillia during processing of dry-fermented meat products (Núñez et al., 2015). In the present study, the occurrence of *D. hansenii* seemed to correlate with the presence of ethyl isopentanoate, recognized as a yeast-produced fruity and green aroma in fermented sausages (Flores et al., 2015).

To the authors' knowledge, this study reports the first detection of *G. hyderabadensis* in *Ciauscolo* salami and more generally in fermented sausages. A paucity of data is available on the occurrence of *Glomus* in the food environment since this mycorrhizal fungus is commonly associated with rhizosphere soils (Rani et al., 2004). Hence, further research

is needed to better clarify the source (e.g. raw materials, food environment) and the role of *G. hyderabadensis* in fermented meat products including *Ciauscolo* salami, the presence of this species has been associated with ethyl hexanoate, a yeast-derived ester compound conferring pear, flowery and sweet aromas to fermented sausages (Flores et al., 2015).

K. zeylanoides (formerly known to as *Candida zeylanoides*) has also been detected at t20 in both the analyzed batches, with batch-dependent differences in terms of relative abundances. Again, to the authors' knowledge, *K. zeylanoides* has never been detected in *Ciauscolo* salami, though this yeast has already been found in *'Nduja* of *Spilinga*, a further PGI spreadable Italian salami (Giarratana et al., 2014). Members of the genus *Candida* have also been reported in "*salsiccia sotto sugna*", a typical salami manufactured in Southern Italy (Gardini et al., 2016).

Regarding small volatile compounds, the detection of spices-derived components throughout the whole ripening process is in accordance with the results previously reported by other authors about different Italian salami (Bianchi et al., 2007; Moretti at al., 2004). The presence of the major aliphatic sulphur compounds as allyl methyl sulphide and diallyl disulphide stably detected in *Ciauscolo* salami could likely been explained by allicin decomposition from the added garlic. This latter hypothesis is supported by the results of Jerković et al. (2010) collected on *Milano* salami, where allicin-derived compounds were recognized to characterize the fresh garlic smell of the end product. Among compounds originated from oxidation of fatty acids (linear aliphatic alcohols and aldehydes), hexanal was the most abundant in *Ciauscolo*, thus confirming the results already observed in other kind of Italian salami (Bianchi et al., 2007; Moretti et al., 2004).

A few aroma components among those several identified could be directly related to microbial activity. Branched chain alcohols originating from amino acid catabolism (3-methyl-1-butanol, 2-methyl-1-butanol) were undoubtedly the most represented (Chaves-López et al., 2014; Janssens et al., 2012). In addition, ethyl esters of isopentanoic, hexanoic (the most abundant), and octanoic acids were recognized as typical markers of microbial activity, as well. According to Bianchi et at. (2007) and Lorenzo et al. (2013), several yeasts, molds and bacteria usually occurring in cured meat could contribute to fruity notes of salami aromas through their esterifying activities. Interestingly, in the samples analyzed in this study a marked decrease of the main lipid oxidation product (hexanal) came with an increase of hexanol, probably due to the reducing conditions inside the product.

5. Conclusions

The results overall collected in the present investigation allowed previously undetected species in *Ciauscolo* salami to be identified. The two independent manufacturing trials (batch 1 and batch 2) at an artisan plant located in the place of

origin of this specialty salami showed the stable presence of the same core bacterial biota that, in the end product, consisted of *L. algidus*, *L. carnosum* and *L. sakei*. Moreover, a core mycobiota composed by *D. hansenii G. hyderabadensis*, *T. washingtonensis*, and *K. zeylanoides* was evidenced. Among the detected species characterizing the microbiota of the model *Ciauscolo* salami, some are those typically associated with fermented sausages whereas other occur with neatly lower frequency in these products, where their role during meat fermentation has still to be clarified. Despite the detection of core bacterial and eumycete species, differences between batches were evidenced in terms of relative abundances, thus highlighting the well-known variability of the production method and artisan manufacturing. Differences also emerged from volatile compounds detection, which resulted to be strongly influenced by the addition of spices to the meat batter. Of note, the absence of foodborne pathogens as *C. botulinum*, *Salmonella* spp. and *Listeria monocytogenes* in all the samples. Further metagenomic analyses carried out onto a more ample number of manufactures from different plants located in the PGI production area are needed to depict a more exhaustive picture of the microbiota occurring in *Ciauscolo* salami, where a higher complexity than that previously described was disclosed by the present study. A deeper knowledge of the microbiota of *Ciauscolo*, as well as other artisan high-value meat-based productions, could be helpful for food business operators and even researchers to improve quality and safety of fermented sausages.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURE CAPTIONS

Figure 1. Slice of ready-to-eat *Ciauscolo* salami produced without the use of starter cultures and nitrates/nitrites.

Figure 2. Incidence of the microbiota taxonomic groups detected by sequencing. Only OTUs with an incidence above 0.2% in at least two samples are shown. Samples are labeled according to time (0, 5, 10 and 20 days).

Figure 3. Incidence of the mycobiota taxonomic groups detected by sequencing. Only OTUs with an incidence above 0.2% in at least two samples are shown. Samples are labeled according to time (0, 5, 10 and 20 days).

Figure 4. Spearman's correlation between microbiota and mycobiota. Only significant associations are shown ($P < 0.05$).

The intensity of the colors represents the degree of correlation between the fungal and bacterial OTUs, as measured by Spearman's correlation, where the blue color represents a positive degree of correlation and red a negative correlation.

Figure 5. Heatplot of the abundances of KEGG genes presumptively belonging to carbohydrate, amino acid and lipid) metabolism pathways in the sausages from batch A (upper red bar) and from batch B (upper blue bar) and.

Rows and columns are clustered by means of Ward linkage hierarchical clustering. The intensity of the colors represents the degree of correlation between the samples and KEGG genes

Credit author statement

Luca Belleggia: Conceptualization, formal analysis. **Vesna Milanović**: formal analysis. **Ilario Ferrocino**: Writing - Review & Editing, formal analysis. **Luca Cocolin**: Writing. **M. Naceur Haouet**: formal analysis. **Stefania Scuota**: formal analysis. **Antonietta Maoloni**: formal analysis. **Cristiana Garofalo**: formal analysis. **Federica Cardinali**: Writing. **Lucia Aquilanti**: Conceptualization, Writing - Review & Editing. **Massimo Mozzon**: Writing, formal analysis. **Roberta Foligni**: formal analysis. **Marina Pasquini**: formal analysis. **Maria Federica Trombetta**: formal analysis. **Francesca Clementi**: Review. **Andrea Osimani**: Conceptualization, Writing - Review & Editing, Supervision, Resources.

Table 1. Physical-chemical parameters of *Ciauscolo* salami during ripening

Batch	Sampling time (days)	pH	a_w	TTA (mL of 0.1N NaOH)	Lactic acid (g/100g)	Acetic acid (g/100g)
1	t0	5.87±0.00^{a}	0.959±0.003^{a}	6.1±0.14^{c}	0.414±0.075^{c}	0.008±0.001^{c}
	t5	5.66±0.01^{b}	0.961±0.000^{a}	5.8±0.00^{c}	0.444±0.073^{c}	0.015±0.002^{b}
	t10	5.68±0.01^{b}	0.950±0.000^{c}	7.0±0.00^{b}	0.541±0.005^{b}	0.020±0.001^{b}
	t20	5.49±0.01^{c}	0.954±0.004^{b}	9.2±0.57^{a}	0.696±0.090^{a}	0.055±0.010^{a}
2	t0	5.86±0.00^{a}	0.960±0.002^{a}	5.8±0.21^{c}	0.445±0.050^{c}	0.008±0.005^{d}
	t5	5.75±0.01^{b}	0.962±0.001^{a}	6.6±0.00^{b}	0.514±0.050^{b}	0.018±0.007^{c}
	t10	5.69±0.01^{c}	0.945±0.007^{c}	6.8±0.28^{b}	0.580±0.077^{b}	0.045±0.023^{b}
	t20	5.69±0.01^{c}	0.954±0.00^{b}	7.9±0.49^{a}	0.614±0.047^{a}	0.059±0.005^{a}

Values are expressed as means ± standard deviation

For each batch, within each column, means with different superscript letters are significantly different ($P < 0.05$).

Table 2. Results of viable counting (log cfu/g) of bacteria and eumycetes in *Ciauscolo* salami during ripening

Batch	Sampling time (days)	Lactic acid bacteria	Coagulase negative cocci	Enterobacteriaceae	Yeasts	Molds
1	t0	2.95±0.28^{c}	2.87±0.04^{c}	3.05±0.08^{a}	3.97±0.24^{c}	2.00±0.00^{a}
	t5	2.73±0.06^{c}	3.21±0.08^{b}	3.03±0.04^{a}	3.95±0.11^{c}	< 2.00^{b}
	t10	7.48±0.00^{b}	3.75±1.01^{a}	2.87±0.12^{b}	5.52±0.23^{a}	< 2.00^{b}
	t20	8.18±0.06^{a}	2.56±0.03^{c}	2.22±0.14^{c}	4.31±0.47^{b}	< 2.00^{b}
2	t0	3.20±0.23^{b}	3.15±0.03^{c}	1.93±0.09^{a}	3.81±0.06^{c}	< 2.00^{b}
	t5	2.97±0.04^{c}	3.42±0.12^{b}	1.86±0.25^{a}	3.98±0.25^{b}	< 2.00^{b}
	t10	3.25±0.31^{b}	2.89±0.62^{c}	1.67±0.24^{b}	4.06±0.14^{b}	< 2.00^{b}
	t20	7.55±0.03^{a}	3.85±0.13^{a}	1.42±0.17^{c}	5.74±0.37^{a}	< 2.00^{b}

Values are expressed as means ± standard deviation.

For each batch, within each column, means with different superscript letters are significantly different ($P < 0.05$).

Table 3. Volatile compounds (mean ± standard deviation [b]) detected in *Ciauscolo* salami during ripening

Peak #[a]	RI	Name	Class	Batch 1 – Ripening time				Batch 2 – Ripening time			
				t0	t5	t10	t20	t0	t5	t10	t20
2		allyl methyl sulfide	SU	61 ± 14^{ab}	93 ± 6^{a}	47 ± 11^{b}	58 ± 6^{ab}	34 ± 2	45 ± 4	40 ± 3	37 ± 37
4		3-methyl-1-butanol	AL	161 ± 8^{a}	124 ± 0^{b}	128 ± 1^{b}	67 ± 5^{c}	168 ± 28^{a}	98 ± 5^{b}	103 ± 4^{b}	111 ± 1^{ab}
5		2-methyl-1-butanol	AL	78 ± 7^{a}	62 ± 0^{ab}	52 ± 9^{b}	43 ± 2^{b}	57 ± 29	45 ± 4	49 ± 1	58 ± 3
6		1-pentanol	AL	46 ± 4^{b}	59 ± 5^{ab}	77 ± 13^{a}	67 ± 5^{ab}	55 ± 43	57 ± 1	46 ± 16	25 ± 5
7	804	hexanal	AD	833 ± 21^{a}	1120 ± 154^{a}	320 ± 27^{b}	435 ± 85^{b}	262 ± 195	536 ± 24	237 ± 26	189 ± 16
8	859	ethyl isopentanoate	ES	2 ± 0^{c}	4 ± 3^{bc}	13 ± 3^{a}	11 ± 0^{ab}	7 ± 3^{b}	3 ± 1^{b}	6 ± 2^{b}	18 ± 2^{a}
9	865	diallyl sulfide	SU	6 ± 2	6 ± 2	6 ± 2	4 ± 1	7 ± 1	7 ± 1	8 ± 2	8 ± 0
10	874	hexanol	AL	11 ± 1^{b}	24 ± 10^{b}	425 ± 100^{a}	330 ± 24^{a}	166 ± 223	129 ± 1	249 ± 156	161 ± 76
11	902	heptanal	AD	26 ± 2	28 ± 7	30 ± 4	38 ± 1	14 ± 6^{ab}	34 ± 4^{a}	21 ± 8^{ab}	12 ± 2^{b}
12	921	allyl methyl disulfide	SU	4 ± 3	7 ± 4	14 ± 11	7 ± 2	2 ± 1	15 ± 5	18 ± 2	18 ± 6
13	933	α-thujene	MH	169 ± 9	183 ± 5	200 ± 32	211 ± 15	660 ± 56	633 ± 5	651 ± 91	786 ± 12
14	941	α-pinene	MH	130 ± 2	132 ± 11	154 ± 29	155 ± 9	653 ± 49	716 ± 53	733 ± 52	817 ± 5
15	956	camphene	MH	5 ± 0^{b}	6 ± 0^{b}	6 ± 1^{a}	7 ± 0^{a}	24 ± 1	24 ± 1	24 ± 2	28 ± 0
A	975	heptanol	AL	2 ± 1	1 ± 1	6 ± 3	9 ± 4	5 ± 5	5 ± 0	7 ± 7	3 ± 0
16	979	sabinene	MH	266 ± 1	222 ± 68	230 ± 51	206 ± 19	844 ± 74	1125 ± 234	1161 ± 58	1267 ± 44
17	982	β-pinene	MH	318 ± 22	300 ± 39	327 ± 81	314 ± 16	1100 ± 33	1249 ± 114	1238 ± 27	1303 ± 28
18	986	2,3-octanedione	KE	72 ± 17^{ab}	138 ± 30^{a}	20 ± 1^{b}	22 ± 6^{b}	26 ± 14	43 ± 2	20 ± 9	13 ± 3
19	993	myrcene	MH	76 ± 4	76 ± 10	74 ± 17	70 ± 2	238 ± 11	276 ± 35	271 ± 12	307 ± 14
20	999	ethyl hexanoate	ES	50 ± 7	50 ± 17	60 ± 1	74 ± 19	84 ± 24	62 ± 2	53 ± 6	47 ± 4
21	1003	octanal	AD	12 ± 0	11 ± 2	13 ± 8	13 ± 5	3 ± 2	14 ± 0	10 ± 4	3 ± 3
22	1007	α-phellandrene	MH	18 ± 2	21 ± 1	18 ± 5	17 ± 0	54 ± 5	45 ± 2	47 ± 12	65 ± 3
23	1014	3-carene	MH	2 ± 0	2 ± 0	2 ± 0	3 ± 0	6 ± 0	6 ± 0	6 ± 1	7 ± 0
24	1021	α-terpinene	MH	49 ± 5	54 ± 1	59 ± 5	62 ± 4	189 ± 6	171 ± 8	173 ± 31	223 ± 1
25	1029	p-cymene	MH	23 ± 2	19 ± 2	26 ± 3	27 ± 1	37 ± 1^{b}	51 ± 7^{a}	47 ± 3^{ab}	47 ± 2^{ab}
26	1035	limonene	MH	846 ± 29	763 ± 102	773 ± 102	734 ± 6	1939 ± 4	2219 ± 288	2211 ± 99	2380 ± 114
27	1043	(Z)-β-ocimene	MH	5 ± 1^{b}	5 ± 1^{b}	3 ± 1^{b}	9 ± 0^{a}	12 ± 1	10 ± 1	10 ± 4	15 ± 1

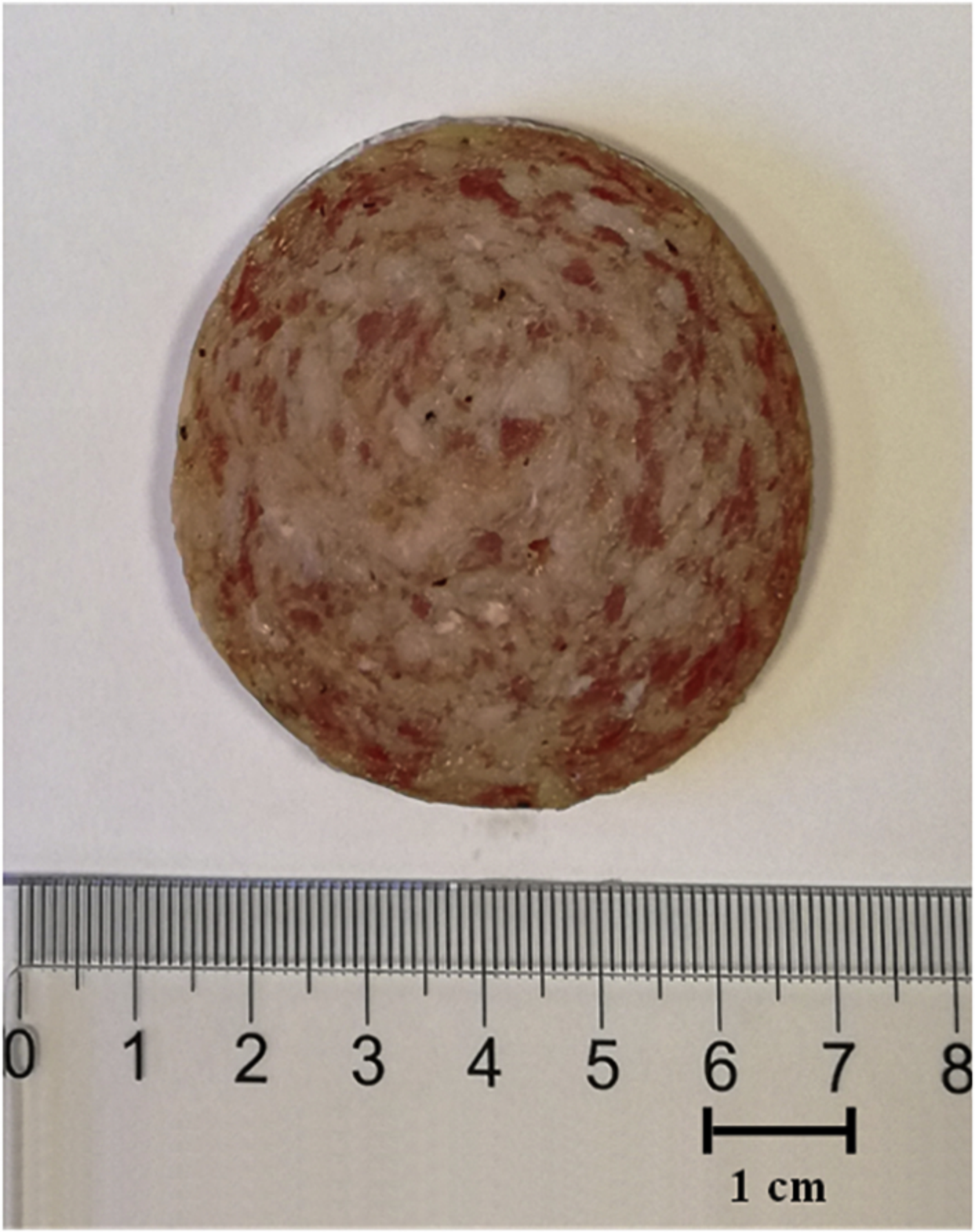
28	1064	γ-terpinene	MH	93 ± 5	103 ± 4	107 ± 13	113 ± 4	331 ± 13	302 ± 10	301 ± 50	389 ± 6
29	1073	cis-sabinene hydrate	OM	11 ± 1	6 ± 2	6 ± 1	7 ± 4	11 ± 3	16 ± 4	16 ± 1	15 ± 0
30	1083	diallyl disulfide	SU	50 ± 25	36 ± 21	35 ± 20	16 ± 4	41 ± 4	84 ± 29	63 ± 19	68 ± 14
31	1091	terpinolene	MH	31 ± 1	35 ± 7	35 ± 0	33 ± 2	121 ± 16	109 ± 0	106 ± 27	163 ± 1
32	1102	unidentified	OM	15 ± 4	12 ± 4	8 ± 1	8 ± 2	7 ± 4^{b}	17 ± 0^{a}	13 ± 1^{ab}	14 ± 0^{ab}
33	1105	unidentified	AL	19 ± 2	20 ± 6	23 ± 3	23 ± 1	20 ± 16	32 ± 6	21 ± 9	10 ± 1
34	1184	1-terpinen-4-ol	OM	13 ± 0	10 ± 1	10 ± 2	9 ± 0	11 ± 1^{b}	15 ± 1^{a}	14 ± 1^{ab}	16 ± 0^{a}
35	1198	ethyl octanoate	ES	3 ± 0	3 ± 0	3 ± 2	4 ± 1	3 ± 0	5 ± 2	2 ± 2	2 ± 0
37	1345	δ-elemene	SH	10 ± 0	8 ± 2	8 ± 2	7 ± 1	7 ± 1	9 ± 2	8 ± 0	9 ± 1
38	1357	α-cubebene	SH	12 ± 1	8 ± 2	9 ± 2	8 ± 1	8 ± 0	13 ± 4	11 ± 0	11 ± 1
39	1375	ylangene	SH	3 ± 0	2 ± 1	3 ± 1	2 ± 0	2 ± 0^{b}	3 ± 1^{a}	3 ± 0^{ab}	3 ± 0^{ab}
40	1384	α-copaene	SH	100 ± 0	79 ± 18	87 ± 20	74 ± 7	76 ± 4	117 ± 37	96 ± 4	94 ± 7
41	1398	β-elemene	SH	5 ± 0	4 ± 1	4 ± 1	3 ± 1	3 ± 1	6 ± 2	4 ± 0	4 ± 1
42	1416	β-caryophillene	SH	2 ± 0	2 ± 0	2 ± 1	2 ± 0	1 ± 1	2 ± 0	2 ± 0	2 ± 0
43	1418	α-gurjunene	SH	1 ± 0	2 ± 1	2 ± 0	1 ± 1	1 ± 1	2 ± 1	2 ± 1	2 ± 1
44	1430	β-copaene	SH	318 ± 2	253 ± 50	254 ± 65	214 ± 34	222 ± 14	345 ± 94	286 ± 1	290 ± 21
45	1438	germacrene D	SH	6 ± 0^{a}	5 ± 1^{ab}	5 ± 1^{ab}	4 ± 1^{b}	3 ± 1	5 ± 2	4 ± 0	4 ± 0
46	1460	allo-aromadendrene	SH	3 ± 0	2 ± 1	2 ± 0	2 ± 0	2 ± 0	3 ± 1	2 ± 0	2 ± 0
47	1464	α-caryophyllene	SH	12 ± 2	9 ± 2	9 ± 2	8 ± 1	7 ± 1	12 ± 4	10 ± 0	10 ± 1
48	1482	β-cadinene	SH	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0
49	1485	γ-muurolene	SH	1 ± 0	1 ± 0	1 ±0	1 ± 0	0 ± 0	1 ± 0	1 ± 0	1 ± 0
50	1496	β-guaiene	SH	2 ± 0	1 ± 0	1 ± 0	1 ± 0	2 ± 0	2 ± 1	1 ± 0	1 ± 0
51	1504	α-selinene	SH	2 ± 0	1 ± 0	1 ± 0	1 ± 0	1 ± 0	2 ± 1	1 ± 0	1 ± 0
52	1507	α-muurolene	SH	3 ± 0	2 ± 1	2 ± 0	2 ± 1	2 ± 0	2 ± 0	2 ± 0	2 ± 1
53	1512	unidentified	SH	12 ± 1	9 ± 3	10 ± 3	8 ± 2	7 ± 1	12 ± 3	9 ± 1	9 ± 1
54	1527	δ-cadinene	SH	11 ± 0	9 ± 2	8 ± 2	7 ± 1	7 ± 1	10 ± 2	9 ± 1	10 ± 1
55	1536	cadina-1,4-diene	SH	3 ± 0	2 ± 1	2 ± 0	2 ± 0	2 ± 0	2 ± 1	2 ± 0	2 ± 0

[a]Peak number according to retention time.
[b]Chromatographic peak areas (arbitrary units $\times 10^4$).
Means with different superscripts in the same row and for the same batch are different at probability level of 0.95.

RI, experimental Kovats Retention Index calculated for a DB-5 type capillary column. SU, sulphur compound. AL, alcohol. AD, aldehyde. ES, ester. MH, monoterpene hydrocarbon. KE, ketone. OM, oxygenated monoterpene. SH, sesquiterpene hydrocarbon.

Highlights

- The microbiota of *Ciauscolo* salami was studied through high-throughput sequencing
- *L. algidus, L. carnosum* and *L. sakei* constituted the core bacterial biota
- *D. hansenii, G. hyderabadensis, K. zeylanoides* prevailed among eumycetes
- Volatile profiles were dominated by the spices-derived components
- A still undisclosed biodiversity in *Ciauscolo* salami was evidenced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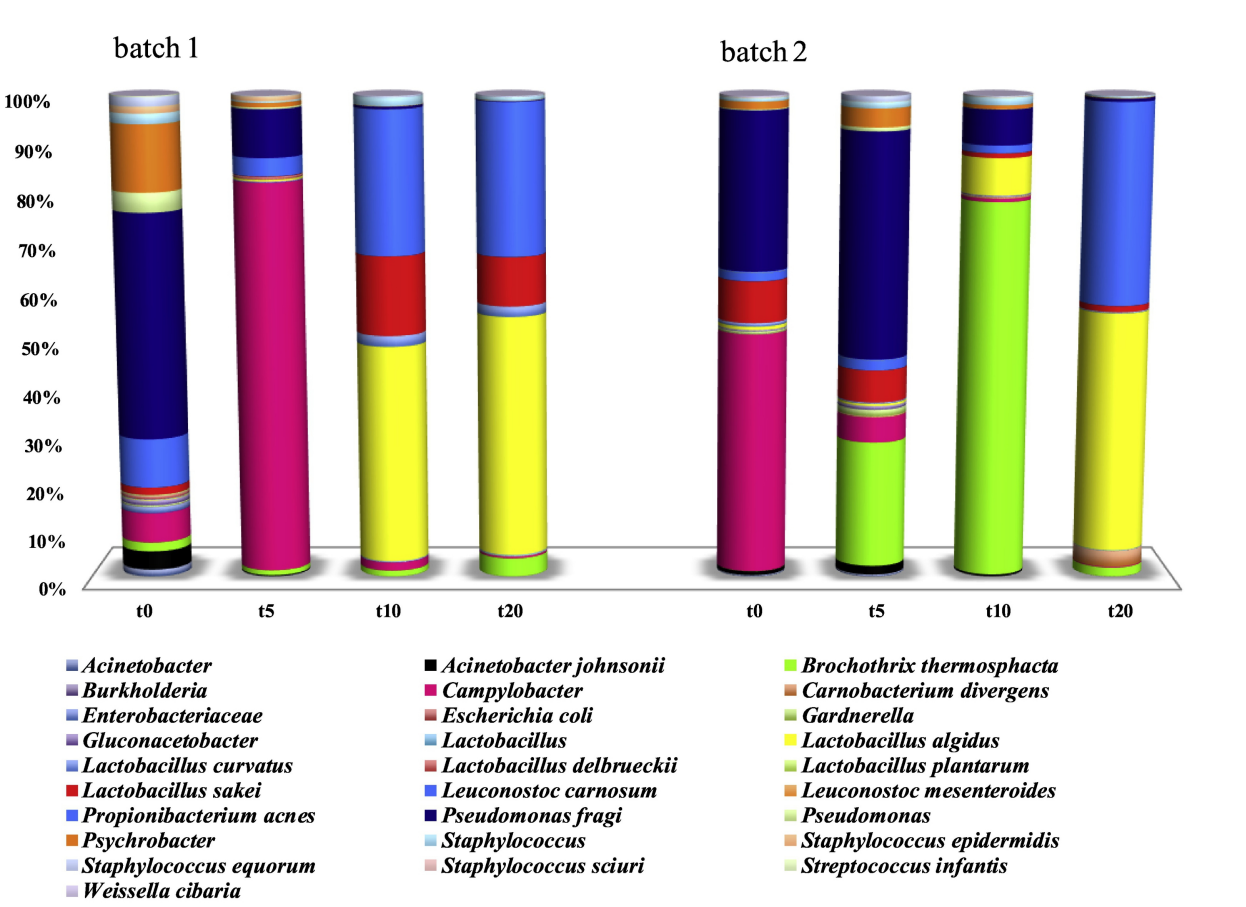
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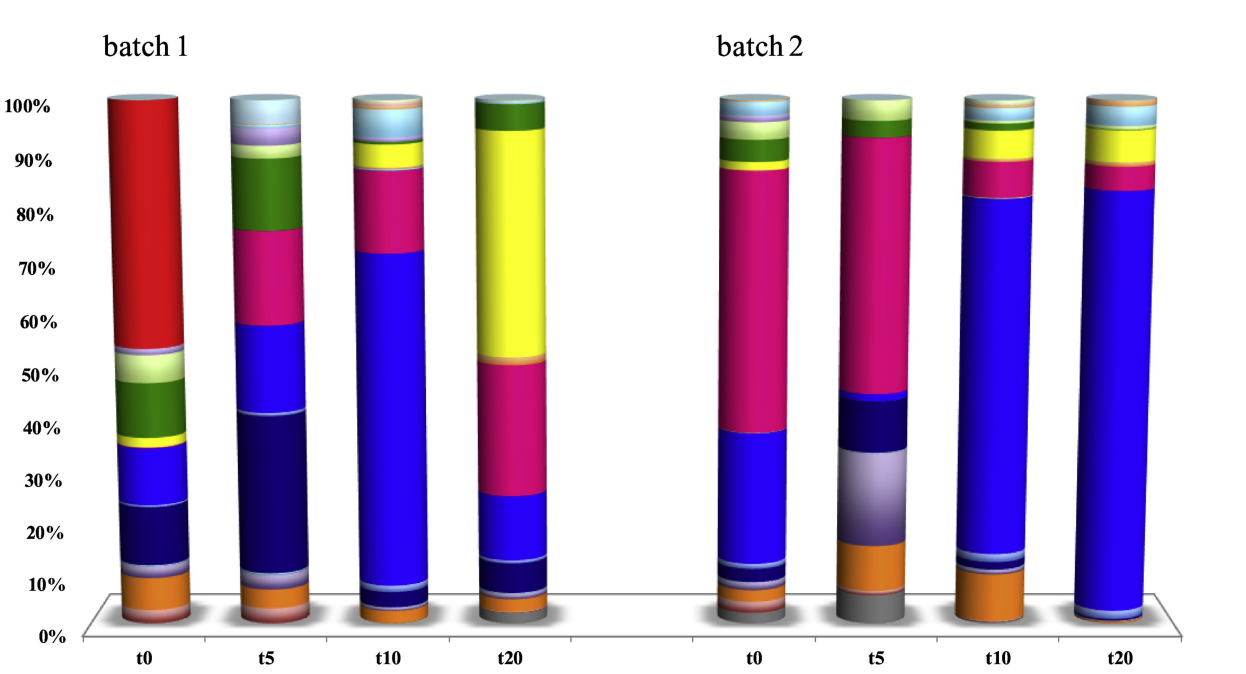
Acinetobacter johnsonii
Brochothrix thermosphacta
Burkholderia
Campylobacter
Carnobacterium divergens
Enterobacteriaceae
Escherichia coli
Gardnerella
Gluconacetobacter
Lactobacillus
Lactobacillus algidus
Lactobacillus curvatus
Lactobacillus delbrueckii
Lactobacillus plantarum
Lactobacillus sakei
Leuconostoc carnosum
Leuconostoc mesenteroides
Propionibacterium acnes
Pseudomonas fragi
Pseudomonas
Psychrobacter
Staphylococcus
Staphylococcus epidermidis
Staphylococcus equorum
Staphylococcus sciuri
Streptococcus infantis
Weissella cibaria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batch 2
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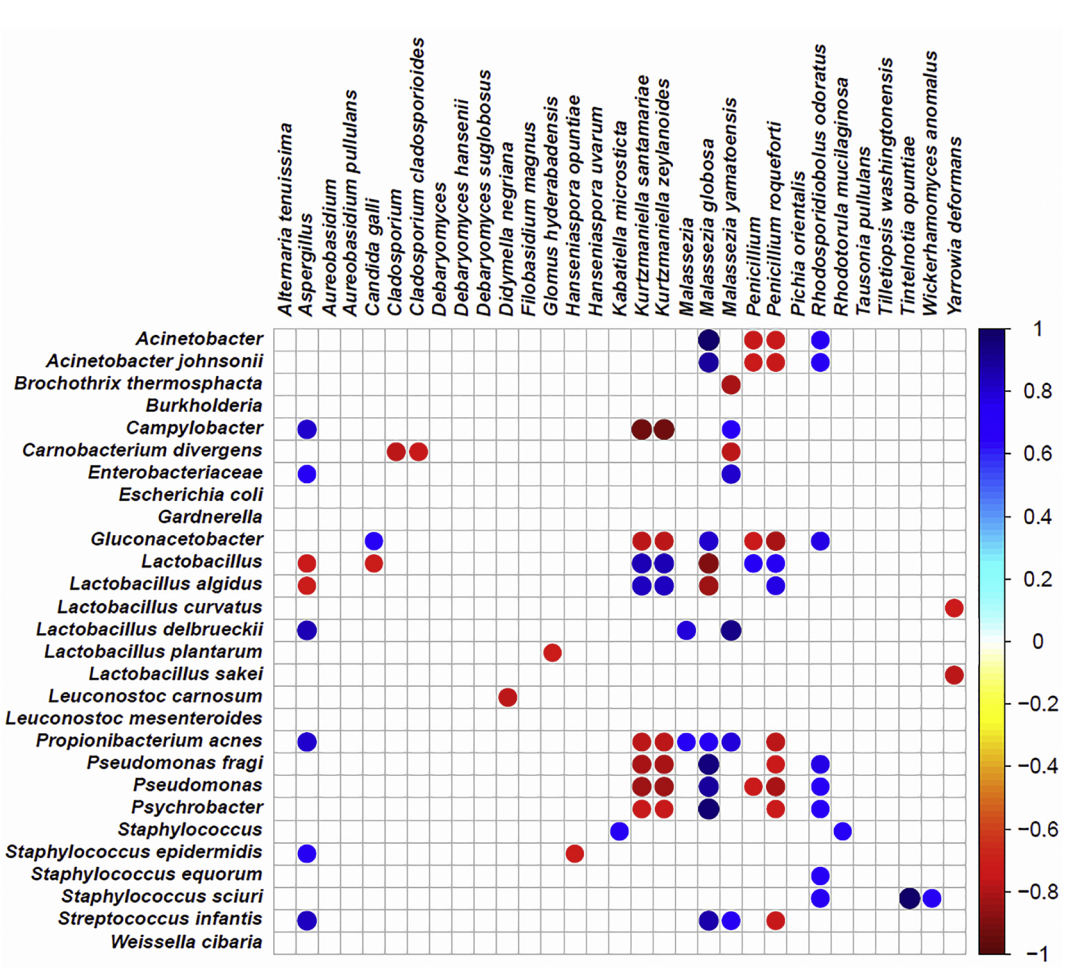
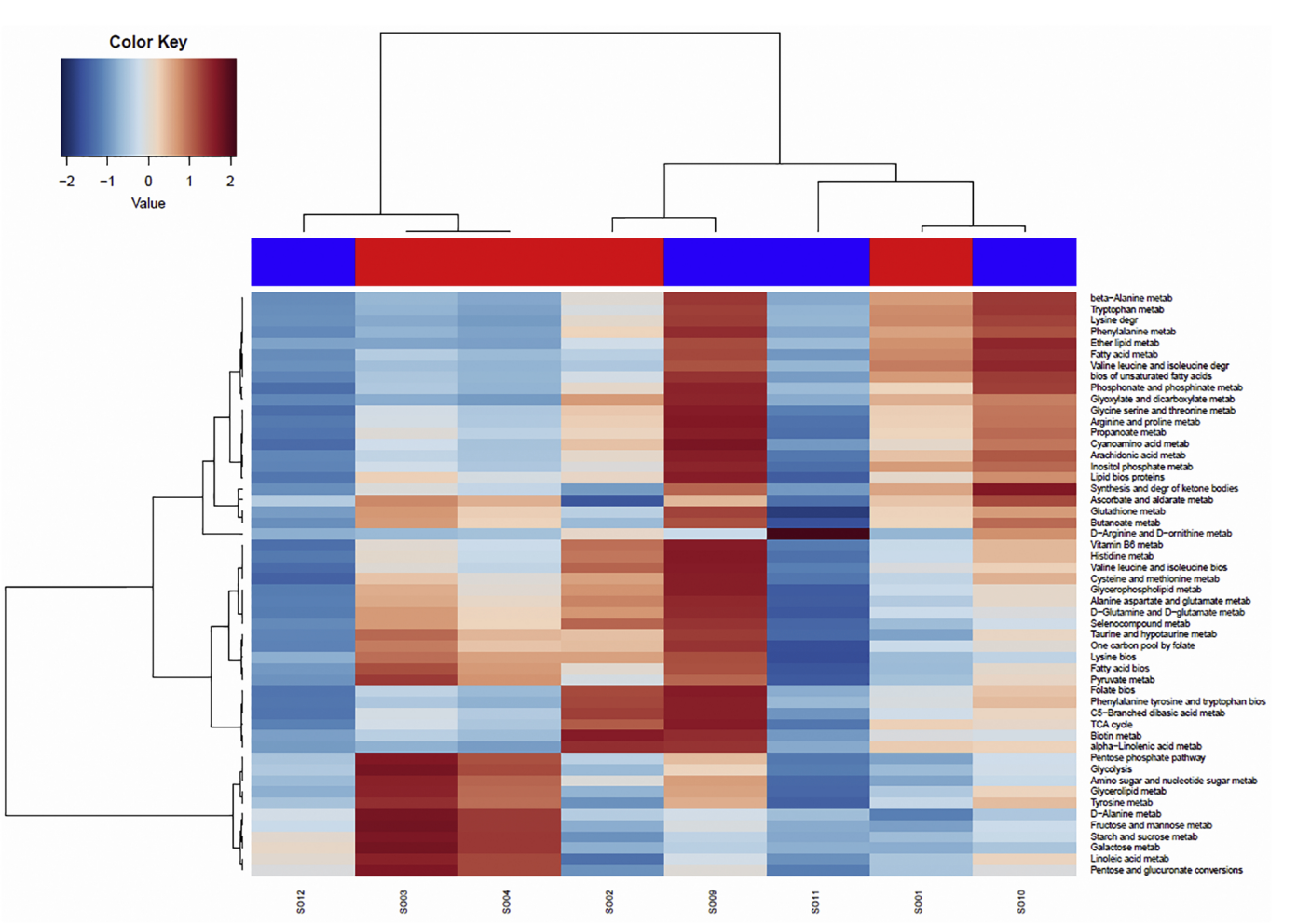
80%
70%
60%
50%
40%
30%
20%
10%
0%
t0
t5
t10
t20
t0
t5
t10
t20
Alternaria tenuissima
Aspergillus
Aureobasidium
Candida galli
Cladosporium
Cladosporium cladosporioides
Debaryomyces
Debaryomyces hansenii
Didymella negriana
Glomus hyderabadensis
Hanseniaspora uvarum
Kurtzmaniella santamariae
Kurtzmaniella zeylanoides
Malassezia
Malassezia globosa
Malassezia yamatoensis
Penicillium
Penicillium roqueforti
Rhizopus oryzae
Rhodotorula mucilaginosa
Tausonia pullulans
Tilletiopsis washingtonensis
Yarrowia deformans


Alternaria tenuissima
Aspergillus
Aureobasidium
Aureobasidium pullulans
Candida galli
Cladosporium
Cladosporium cladosporioides
Debaryomyces
Debaryomyces hansenii
Debaryomyces suglobosus
Didymella negriana
Filobasidium magnus
Glomus hyderabadensis
Hanseniaspora opuntiae
Hanseniaspora uvarum
Kabatiella microsticta
Kurtzmaniella santamariae
Kurtzmaniella zeylanoides
Malassezia
Malassezia globosa
Malassezia yamatoensis
Penicillium
Penicillium roqueforti
Pichia orientalis
Rhodosporidiobolus odoratus
Rhodotorula mucilaginosa
Tausonia pullulans
Tilletiopsis washingtonensis
Tintelnotia opuntiae
Wickerhamomyces anomalus
Yarrowia deformans
Acinetobacter
Acinetobacter johnsonii
Brochothrix thermosphacta
Burkholderia
Campylobacter
Carnobacterium divergens
Enterobacteriaceae
Escherichia coli
Gardnerella
Gluconacetobacter
Lactobacillus
Lactobacillus algidus
Lactobacillus curvatus
Lactobacillus delbrueckii
Lactobacillus plantarum
Lactobacillus sakei
Leuconostoc carnosum
Leuconostoc mesenteroides
Propionibacterium acnes
Pseudomonas fragi
Pseudomonas
Psychrobacter
Staphylococcus
Staphylococcus epidermidis
Staphylococcus equorum
Staphylococcus sciuri
Streptococcus infantis
Weissella cibaria
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1


Color Key
−2
−1
0
1
2
Value
beta−Alanine metab
Tryptophan metab
Lysine degr
Phenylalanine metab
Ether lipid metab
Fatty acid metab
Valine leucine and isoleucine degr
bios of unsaturated fatty acids
Phosphonate and phosphinate metab
Glyoxylate and dicarboxylate metab
Glycine serine and threonine metab
Arginine and proline metab
Propanoate metab
Cyanoamino acid metab
Arachidonic acid metab
Inositol phosphate metab
Lipid bios proteins
Synthesis and degr of ketone bodies
Ascorbate and aldarate metab
Glutathione metab
Butanoate metab
D−Arginine and D−ornithine metab
Vitamin B6 metab
Histidine metab
Valine leucine and isoleucine bios
Cysteine and methionine metab
Glycerophospholipid metab
Alanine aspartate and glutamate metab
D−Glutamine and D−glutamate metab
Selenocompound metab
Taurine and hypotaurine metab
One carbon pool by folate
Lysine bios
Fatty acid bios
Pyruvate metab
Folate bios
Phenylalanine tyrosine and tryptophan bios
C5−Branched dibasic acid metab
TCA cycle
Biotin metab
alpha−Linolenic acid metab
Pentose phosphate pathway
Glycolysis
Amino sugar and nucleotide sugar metab
Glycerolipid metab
Tyrosine metab
D−Alanine metab
Fructose and mannose metab
Starch and sucrose metab
Galactose metab
Linoleic acid metab
Pentose and glucuronate conversions
SO12
SO03
SO04
SO02
SO09
SO11
SO01
SO10